EXHIBIT A



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Helleday

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(45) **Date of Patent:**

Oct. 14, 2014

(54) USE OF RNAI INHIBITING PARP ACTIVITY FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF **CANCER**

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§ 371 (c)(1),

(2), (4) Date: Sep. 15, 2006

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	C12N 15/113	(2010.01)
	A61K 31/5517	(2006.01)

(52) U.S. Cl. CPC C07D 487/06 (2013.01); A61K 38/005 (2013.01); C12N 15/1137 (2013.01); A61K 31/5517 (2013.01); C12Y 204/0203 (2013.01);

> C12N 2310/14 (2013.01) USPC **514/258.1**; 514/299; 514/388

(58)Field of Classification Search

See application file for complete search history.

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Primary Examiner — James D Anderson (74) Attorney, Agent, or Firm — Bozicevic, Field & Francis LLP; Pamela J. Sherwood

(57)**ABSTRACT**

The present invention relates to the use of an agent that inhibits the activity of an enzyme that mediates repair of a DNA strand break in the manufacture of a medicament for the treatment of diseases caused by a defect in a gene that mediates homologous recombination.

1 Claim, 20 Drawing Sheets

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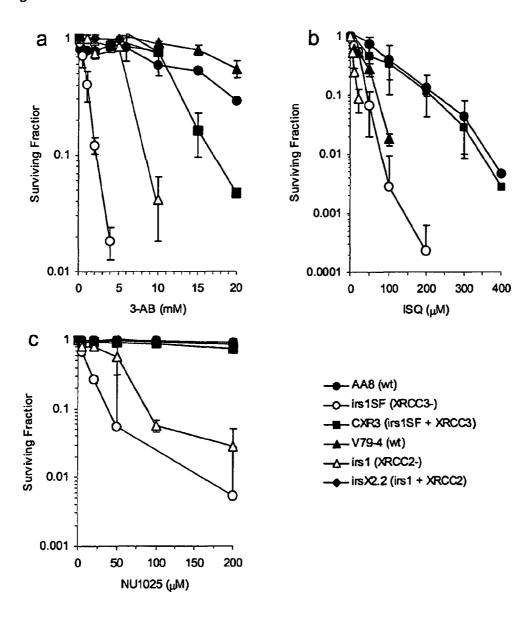
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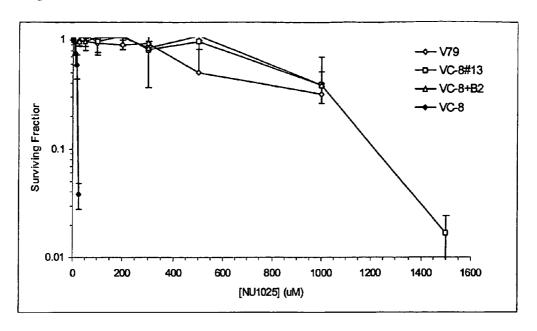
Figure 1.



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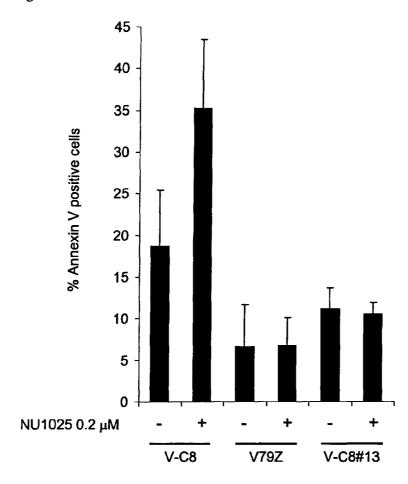
Figure 2.



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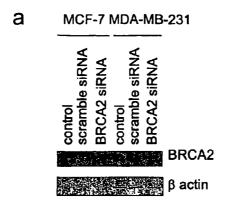
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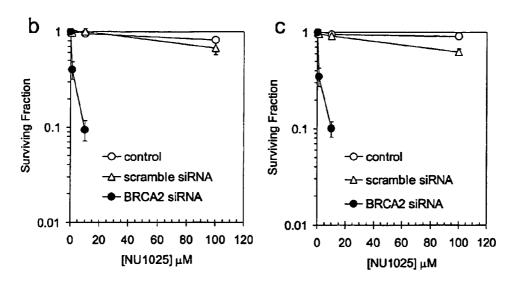


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Figure 4

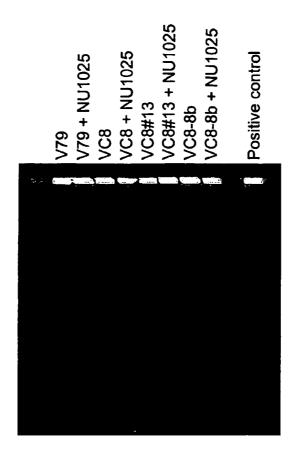




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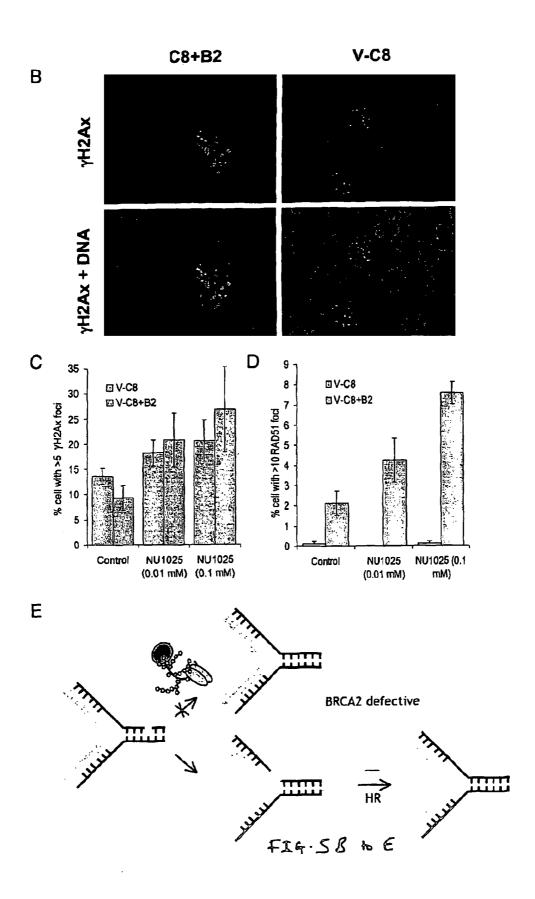
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Figure 5A



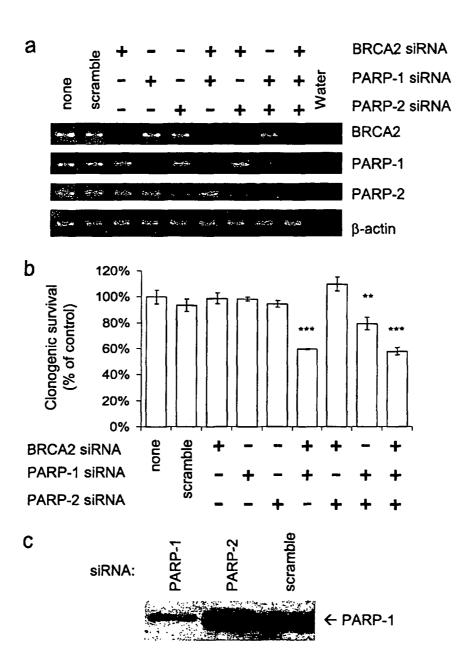
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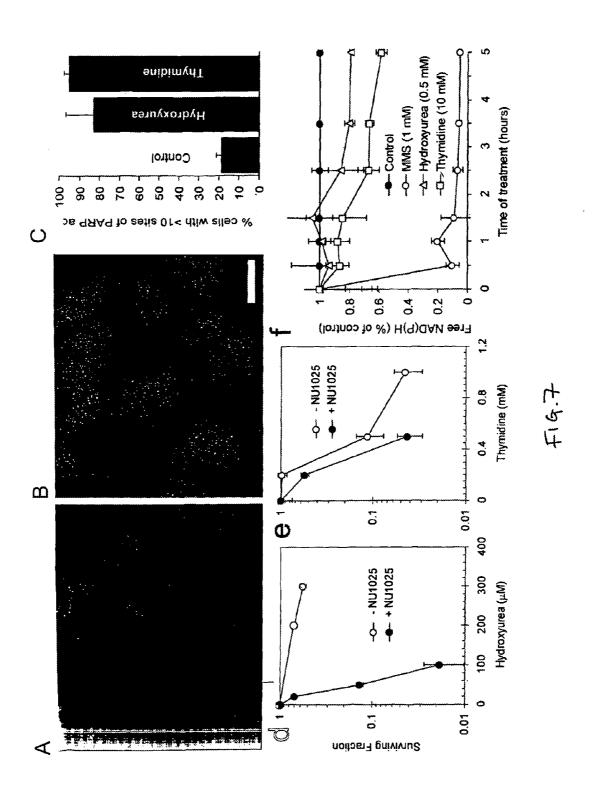
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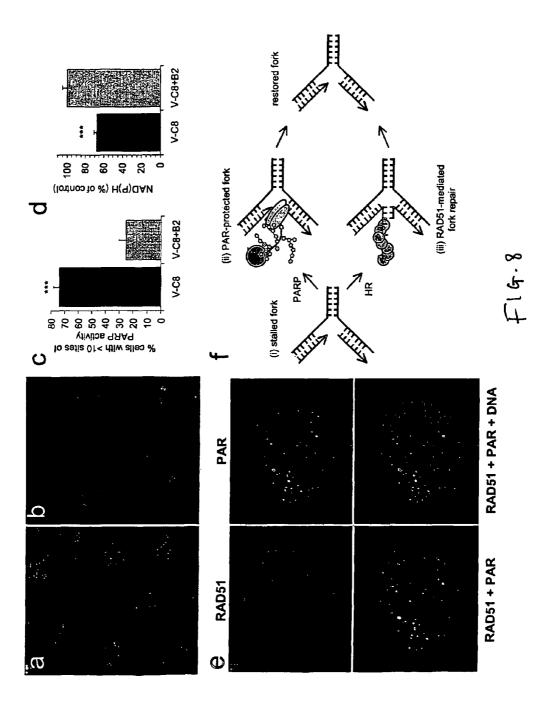
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FIGURE 9

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Figure 10

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Figure 11

1 tgggactggt cgcctgactc ggcctgcccc agcctctgct tcaccccact ggtggccaaa 61 tagecgatgt etaateccee acaeaagete atecceggee tetgggattg ttgggaatte 121 tetecetaat teaegeetga ggeteatgga gagttgetag acetgggaet geeetgggag 181 gegeacacaa eeaggeeggg tggeageeag gaceteteee atgteeetge ttttettgge 241 catggeteca aageegaage cetgggtaca gaetgaggge cetgagaaga agaagggeeg 301 gcaggcagga agggaggagg acccetteng etenaceget gaggecetea aggecatace 361 egeagagaag egeataatee gegtggatee aacatgteea eteageagea acceegggae 421 ccaggtgtat gaggactaca actgcaccct gaaccagacc aacatcgaga acaacaacaa 481 caagttetae atcatecage tgeteeaaga cageaacege ttetteacet getggaaceg 541 ctggggccgt gtgggagagg tcggccagtc aaagatcaac cacttcacaa ggctagaaga 601 tgcaaagaag gactttgaga agaaatttcg ggaaaagacc aagaacaact gggcagagcg 661 ggaccacttt gtgtctcacc cgggcaagta cacacttatc gaagtacagg cagaggatga 721 ggcccaggaa gctgtggtga aggtggacag aggcccagtg aggactgtga ctaagcgggt 781 geagecetge teeetggace eagecaegea gaageteate aetaacatet teageaagga 841 gatgttcaag aacaccatgg ccctcatgga cctggatgtg aagaagatgc ccctgggaaa 901 gctgagcaag caacagattg cacggggttt cgaggccttg gaggcgctgg aggaggccct 961 gaaaggcccc acggatggtg gccaaagcct ggaggagctg tcctcacact tttacaccgt 1021 catecegeae aactteggee acagecagee eeegeecate aatteeeetg agettetgea 1081 ggccaagaag gacatgctgc tggtgctggc ggacatcgag ctggcccagg ccctgcaggc 1141 agtetetgag caggagaaga eggtggagga ggtgccacac eccetggace gagactacca 1201 getteteaag tgecagetge agetgetaga etetggagea eetgagtaca aggtgataca 1261 gacctactta gaacagactg gcagcaacca caggtgccct acacttcaac acatctggaa 1321 agtaaaccaa gaagggagg aagacagatt ccaggcccac tccaaactgg gtaatcggaa 1381 getgetgtgg catggcacca acatggccgt ggtggccgcc atcctcacta gtgggctccg 1441 catcatgcca cattetggtg ggcgtgttgg caagggcate tactttgcct cagagaacag 1501 caagtcagct ggatatgtta ttggcatgaa gtgtggggcc caccatgtcg gctacatgtt 1561 cctgggtgag gtggccctgg gcagagagca ccatatcaac acggacaacc ccagcttgaa 1621 gageceacet cetggetteg acagtgteat tgeeegagge cacacegage etgateegae 1681 ccaggacact gagttggagc tggatggcca gcaagtggtg gtgccccagg gccagcctgt 1741 gecetgecea gagtteagea geteeacatt eteecagage gagtacetea tetaceagga 1801 gagccagtgt egeetgeget acetgetgga ggtccacete tgagtgeeeg eeetgteeee 1861 cggggtcctg caaggctgga ctgtgatctt caatcatcct gcccatctct ggtaccccta 1921 tatcactect ttttttcaag aatacaatac gttgttgtta actatagtca ccatgctgta 1981 caagateect gaacttatge etectaaetg aaattttgta ttetttgaca eatetgeeca 2041 gtccctctcc tcccagccca tggtaaccag catttgactc tttacttgta taagggcagc 2101 ttttataggt tccacatgta agtgagatca tgcagtgttt gtctttctgt gcctggctta 2161 tttcactcag cataatgtgc accgggttca cccatgtttt cataaatgac aagatttcct

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Figure 12

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1 cgaagatggc ggcgtcgcgt cgctctcagc atcatcacca ccatcatcaa caacagctcc
  61 ageocycecc aggggettca gegeogoogo egecacetec teccecacte ageoctggec
 121 tggccccggg gaccacccca gcctctccca cggccagcgg cctggccccc ttcgcctccc
 181 egeggeaegg cetagegetg ceggaggggg atggeagteg ggateegeec gaeaggeece
 241 gateceegga ecoggitigae ggtaceaget gitigeagtae caceageaea atetgitaceg
 301 tegeogeege teeegtggte coageggttt etactteate tgeogetggg gtegeteeca
 361 acccagoogg cagtggcagt aacaatteac ogtogtoote ttottocccg acttottoct
 421 catetteete tecatectee eetqqateqa qettqqeqqa qaqeeegag geggeeggag
 481 trageageac ageaceactg gggeetgggg cageaggace tgggacaggg gteccageag
 541 tgagcggggc cctacgggaa ctgctggagg cctgtcgcaa tggggacgtg tcccgggtaa
 601 agaggetggt ggacgeggca aacgtaaatg caaaggacat ggccggccgg aagtettete
 661 cectgeactt cgctgeaggt tttggaagga aggatgttgt agaacactta ctacagatgg
 721 gtgctaatgt ccacgctogt gatgatggag gtctcatccc gcttcataat gcctgttctt
 781 ttggccatgc tgaggttgtg agtotgttat tgtgccaagg agctgatcca aatgccaggg
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1081 atgtgaattg ccatgcaagt gatgggcgaa agtcgactcc tttacatcta gcagcgggct
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1441 agottaggga gagattgact tatgaattta aaggtcattc tttactacaa gcagccagag
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1621 aagtgacaga attgttactt agaaaaggag caaatgttaa tgaaaaaaat aaagatttca
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1741 agcatggcgc caagatgaat gcactggaca cccttggtca gactgctttg catagagccg
1801 ccctagcagg ccaectgcag acctgccgcc tectgctgag ttacggctet gaccetteca
1861 teatcheett acaaggette acagcageae agatgggeaa tgaagcagtg cagcagatte
1921 tgagtgtgag ttacggctct gacccctcca tcatctcctt acaaggcttc acagcagcac
1981 agatgggcaa tgaagcagtg cagcagattc tgagtggtca ttcgtagata gtgatcattc
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2101 qtcttcactg tcaacatgaa gagtacacct atacgtactt ctgatgttga ttatcgactc
2161 ttagaggcat ctaaagctgg agacttggaa actgtgaagc aactttgcag ctctcaaaat
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2401 tagagggccg gcattccacg cccttacact tegcagcagg ctacaaccgc gtgtctgttg
2461 tagagtacct gctacaccac ggtgccgatg tccatgccaa agacaagggt ggcttggtgc
2521 cccttcataa tgcctgttca tatggacact atgaggtggc tgagctttta gtaaggcatg
2581 gggettetgt caatgtggeg gaettatgga aatttacece tetecatgaa geageageta
2641 aaggaaagta tgaaatctgc aagctccttt taaaacatgg agcagatcca actaaaaaga
2701 acagagatgg aaatacacct ttggatttgg taaaggaagg agacacagat attcaggact
2761 tactgaaagg ggatgctgct ttgttggatg ctgccaagaa gggctgcctg gcaagagtgc
2821 agaagetetg taccccagag aatatcaact gcagagacac ccagggcaga aattcaaccc
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2941 gagotgatgt taatgeceag gacaagggtg gtttaattce tetteataat geggeatett
3001 atgggcatgt tgacatagcg gctttattga taaaatacaa cacgtgtgta aatgcaacag
3061 ataagtgggc gtttactecc ctccatgaag cageccagaa aggaaggacg cagetgtgcg
3121 controttent agegratggt gragactera cratgaagaa craggaagge ragaegente
3181 tggatctggc aacagctgac gatatcagag ctttgctgat agatgccatg cccccagagg
3241 cettacetae etgetetaaa ceteaggeta etgeagtgag tgeetetetg ateteaceag
3301 categacee etectgeete teggetgeca geageataga caaceteact ggecetttag
3361 cagagitggc cgtaggagga goctocaatg caggggatgg cgccgcggga acagaaagga
3421 aggaaggaga agttgctggt cttgacatga atatcagcca atttctaaaa agccttggcc
3481 ttgaacacct tcgggatatc tttgaaacag aacagattac actagatgtg ttggctgata
3541 tgggtcatga agagttgaaa gaaataggca tcaatgcata tgggcaccgc cacaaattaa
3601 tcaaaqqaqt aqaaaqactc ttaggttggac aacaaggcac caatccttat ttgacttttc
3661 actgtgttaa tcagggaacg attttgctgg atcttgctcc agaagataaa gaatatcagt
3721 cagtggaaga agagatgcaa agtactattc gagaacacag agatggtggt aatgctggcg
3781 qcatcttcaa cagatacaat qtcattcgaa ttcaaaaagt tgtcaacaag aagttgaggg
3841 ageggttetg ceacegacag aaggaagtgt etgaggagaa teacaaccat cacaatgage
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3901 gcatgttgtt tcatggttet cctttcatta atgccattat tcataaaggg tttgatgagc 3961 gacatgcata cataggagga atgtttgggg ccgggattta ttttgctgaa aactcctcaa 4021 aaagcaacca atatgtttat ggaattggag gaggaacagg ctgccctaca cacaaggaca 4081 ggtcatgcta tatatgtcac agacaaatgc tcttctgtag agtgaccett gggaaatcct 4141 ttctgcagtt tagcaccatg aaaatggccc acgcgcctcc agggcaccac tcagtcattg 4201 gtagaccgag cgtcaatggg ctggcatatg ctgaatatgt catctacaga ggagaacagg 4261 catacccaga gtatcttatc acttaccaga tcatgaagcc agaagcccct tcccagaccg 4321 caacagccgc agagcagaag acctagtgaa tgcctgctgg tgaaggccag atcagattc 4381 aacctgggac tggattacag aggattgttt ctaataacaa catcaatatt ctagaagtcc 4441 ctgacagcct agaaataagc tgtttgtctt ctataaagca ttgctatag g
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Figure 12 (continued)

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Figure 13

1 egegeegeet egetageega aacetgeeca geeggtgeee ggeeactgeg eacgeggg 61 acgaegteac gtgcgetecc ggggetggae ggagetggea ggaggggeet tgccagette 121 cgccgccgcg tegtttcagg acccggacgg cggattcgcg ctgcctccgc cgccgcgggg 181 cagccggggg geagggagec cagcgagggg cgegegtggg egeggecatg ggaetgegee 241 ggatccggtg acagcaggga gccaagcggc ccgggccctg agcgcgtctt ctccgggggg 301 cetegecete etgetegegg ggeegggget eetgeteegg ttgetggege tgttgetgge 361 tgtggcggeg gecaggatea tgtcgggteg cegetgegee ggcgggggag cggcctgcgc 421 gagegeegeg geegaggeeg tggageegge egeeegagag etgttegagg egtgeegeaa 481 cggggacgtg gaacgagtca agaggctggt gacgcctgag aaggtgaaca gccgcgacac 541 ggcgggcagg aaatccacce cgctgcactt cgccgcaggt tttgggcgga aagacgtagt 601 tgaatatttg cttcagaatg gtgcaaatgt ccaagcacgt gatgatgggg gccttattcc 661 tetteataat geatgetett ttggteatge tgaagtagte aateteettt tgegacatgg 721 tgcagacccc aatgetegag ataattggaa ttatactcct etceatgaag etgeaattaa 781 aggaaagatt gatgtttgca ttgtgctgtt acagcatgga gctgagccaa ccatccgaaa 841 tacagatgga aggacagcat tggatttagc agatccatct gccaaagcag tgcttactgg 901 tgaatataag aaagatgaac tettagaaag tgecaggagt ggcaatgaag aaaaaatgat 961 ggctetacte acaccattaa atgteaactg ceaegeaagt gatggeagaa agteaactee 1021 attacatttg gcagcaggat ataacagagt aaagattgta cagctgttac tgcaacatgg 1081 agctgatgte catgetaaag ataaaggtga tetggtacca ttacacaatg cetgttetta 1141 tggtcattat gaagtaactg aactttiggt caagcatggt gcctgtgtaa atgcaatgga 1201 ettgtggcaa tteaeteete tteatgagge agettetaag aacagggttg aagtatgtte 1261 tettetetta agttatggtg cagacceaae aetgeteaat tgteacaata aaagtgetat 1321 agacttggct cccacaccac agttaaaaga aagattagca tatgaattta aaggccactc 1381 gttgctgcaa gctgcacgag aagetgatgt tactcgaatc aaaaaacatc tctctctgga 1441 aatggtgaat tteaageate eteaaacaea tgaaacagea ttgeattgtg etgetgeate 1501 tecatatece aaaagaaage aaatatgtga aetgttgeta agaaaaggag caaacateaa 1561 tgaaaagact aaagaattet tgacteetet geaegtggea tetgagaaag eteataatga 1621 tgttgttgaa gtagtggtga aacatgaagc aaaggttaat getetggata atettggtea 1681 gaetteteta cacagagetg catattgtgg teatetacaa acetgeegee tacteetgag 1741 ctatgggtgt gatcctaaca ttatatccct tcagggettt actgetttac agatgggaaa 1801 tgaaaatgta cagcaactcc tccaagaggg tatctcatta ggtaattcag aggcagacag 1861 acaattgctg gaagctgcaa aggctggaga tgtcgaaact gtaaaaaaac tgtgtactgt 1921 teagagtgte aactgeagag acattgaagg gegteagtet acaccactte attttgeage 1981 tgggtataac agagtgteeg tggtggaata tetgetacag catggagetg atgtgeatge 2041 taaagataaa ggaggeettg tacetttgea caatgeatgt tettatggae attatgaagt 2101 tgcagaactt cttgttaaac atggagcagt agttaatgta gctgatttat ggaaatttac 2161 acctttacat gaagcagcag caaaaggaaa atatgaaatt tgcaaacttc tgctccagca 2221 tggtgcagac cctacaaaaa aaaacaggga tggaaatact cctttggatc ttgttaaaga

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2821 tgtgagaage ceaggageea etgeagatge tetetettea ggteeateta geceateaag 2881 cetttetgea geeageagte ttgacaactt atetgggagt tttteagaac tgtetteagt 2941 agttagttca agtggaacag agggtgcttc cagtttggag aaaaaggagg ttccaggagt 3001 agattttagc ataactcaat togtaaggaa tottggactt gagcacctaa tggatatatt 3061 tgagagagaa cagatcactt tggatgtatt agttgagatg gggcacaagg agctgaagga 3121 gattggaatc aatgettatg gacataggea caaactaatt aaaggagteg agagacttat 3181 eteeggacaa eaaggtetta aeceatattt aaetttgaac aectetggta gtggaacaat 3241 tettatagat etgteteetg atgataaaga gttteagtet gtggaggaag agatgeaaag 3301 tacagttega gagcacagag atggaggtea tgcaggtgga atctteaaca gatacaatat 3361 teteaagatt eagaaggttt gtaacaagaa actatgggaa agatacaete aeeggagaaa 3421 agaagtttet gaagaaaace acaaceatge caatgaacga atgetattte atgggtetee 3481 ttttgtgaat geaattatee acaaaggett tgatgaaagg catgegtaca taggtggtat 3541 gtttggaget ggeatttatt ttgetgaaaa etetteeaaa ageaateaat atgtatatgg 3601 aattggagga ggtactgggt gtccagttca caaagacaga tcttgttaca tttgccacag 3661 geagetgete tittgeeggg taacettggg aaagtettte etgeagttea gtgeaatgaa 3721 aatggcacat teteeteeag gteateacte agteaetggt aggeeeagtg taaatggeet 3781 agcattaget gaatatgtta tttacagagg agaacagget tatcetgagt atttaattac 3841 ttaccagatt atgaggeetg aaggtatggt egatggataa atagttattt taagaaacta 3901 attocactga acctaaaatc atcaaagcag cagtggcctc tacgttttac tcctttgctg 3961 aaaaaaaatc atcttgccca caggcctgtg gcaaaaggat aaaaatgtga acgaagttta 4021 acattetgae ttgataaage tttaataatg tacagtgttt tetaaatatt teetgttttt 4081 teageaettt aacagatgee atteeaggtt aaactgggtt gtetgtaeta aattataaac 4141 agagttaact tgaacctttt atatgttatg cattgattct aacaaactgt aatgccctca 4201 acagaactaa ttttactaat acaatactgt gttctttaaa acacagcatt tacactgaat 4261 acaatttcat ttgtaaaact gtaaataaga gettttgtac tageecagta tttatttaca 4321 ttgctttgta atataaatet gttttagaac tgcageggtt tacaaaattt tttcatatgt 4381 attgitcate tataciteat ettacategt eatgattgag tgatetttac atttgattee 4441 agaggetatg tteagttgtt agttgggaaa gattgagtta teagatttaa tttgeegatg 4501 ggagcettta tetgteatta gaaatettte teatttaaga aettatgaat atgetgaaga 4561 tttaatttgt gatacetttg tatgtatgag acacatteca aagageteta actatgatag 4621 gtcctgatta ctaaagaage ttctttactg gcctcaattt ctagetttca tgttggaaaa 4681 ttttctgcag tccttctgtg aaaattagag caaagtgctc ctgtttttta gagaaactaa 4741 atettgetgt tgaacaatta tigtgttett tteatggaac ataagtagga tgttaacatt 4801 tecagggtgg gaagggtaat cetaaateat tteceaatet attetaatta eettaaatet 4861 aaaggggaaa aaaaaaatca caaacaggac tgggtagttt tttatcctaa gtatattttt 4921 teetgttett tttacttggt tttattgetg tatttatage caatetatae ateatgggta 4981 aacttaaccc agaactataa aatgtagttg tttcagtccc cttcaggcct cctgaatggg 5041 caagtgcagt gaaacaggtg cttcctgctc ctgggttttc tctccatgat gttatgccca 5101 attggaaata tgctgtcagt ttgtgcacca tatggtgacc acgcctgtgc tcagtttggc 5161 agetatagaa ggaaatgetg teecataaaa tgecateeet atttetaata taacaetett 5221 ttccaggaag catgettaag catettgtta cagagacata catecattat ggettggcaa 5281 tetettttat tigttgaete tageteeett caaagtegag gaaagatett taeteaetta 5341 atgaggacat tececateae tgtetgtace agtteaeett tattttaegt tttatteagt 5401 etgtaaatta aetggeeett tgeagtaaet tgtacataaa gtgetagaaa ateatgttee 5461 ttgtcctgag taagagttaa tcagagtaag tgcatttctg gagttgtttc tgtgatgtaa 5521 attatgatca ttatttaaga agtcaaatcc tgatcttgaa gtgcttttta tacagctctc 5581 taataattac aaatateega aagteattte ttggaacaca agtggagtat gecaaatttt 5641 atatgaattt tteagattat etaagettee aggttttata attagaagat aatgagagaa 5701 ttaatggggt ttatatttac attatctctc aactatgtag cccatattac tcaccctatg 5761 agtgaatetg gaattgettt teatgtgaaa teattgtggt etatgagttt acaataetge

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- 5821 aaactgtgtt attttatcta aaccattget taatgagtgt gttttteeat gaatgaatat
- 5881 accgtggttc atatgttagc atggcagcat tttcagatag ctttttgttt gttgggaagt
- 5941 tggggttttg gggggagggg gagtattagt acgttgcatg gaatagccta ctttataatg
- 6061 gtgccagtag tactattata cccatcttca gtgtcttact tgtactgtat caaattccat
- 6121 acceteattt aattettaat aaaaetgtte acttgtaaaa aaaaaaaaaa aaaaaaaaaa
- 6181 aaaaaaaaa

Figure 13 (continued)

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Figure 14

1 cgcccgccca gccccggggg cagggaaagc ctaaattacg gaattaccgc gagcaaggag 61 cgcggaatcg gggagcgtcc ggagctagct ggatcctcta ggcaggatgg tgatgggaat 121 ettigeaaat tgtatettet gtttgaaagt gaagtaetta eeteageage agaagaaaaa 181 getacaaact gacattaagg aaaatggegg aaagttttee ttttegttaa ateeteagtg 241 cacacatata atcitagata atgctgatgt tetgagteag taccaactga attetateca 301 aaagaaccac gitcatatig caaacccaga tittatatgg aaatctatca gagaaaagag 361 actettggat gtaaagaatt atgateetta taageeeetg gacateacae caceteetga 421 teagaaggeg ageagttetg aagtgaaaac agaaggteta tgeeeggaca gtgeeacaga 481 ggaggaagae actgtggaac teactgagtt tggtatgeag aatgttgaaa ttecteatet 541 tecteaagat tttgaagttg caaaatataa cacettggag aaagtgggaa tggagggagg 601 ccaggaaget gtggtggtgg agetteagtg ttegegggae teeagggaet gteettteet 661 gatateetea eaetteetee tggatgatgg eatggagaet agaagaeagt ttgetataaa 721 gaaaacctct gaagatgcaa gtgaatactt tgaaaattac attgaagaac tgaagaaaca 781 aggattteta etaagagaae attteacaee tgaageaaee eaattageat etgaacaatt 841 gcaagcattg ettttggagg aagteatgaa tteaageact etgageeaag aggtgagega 901 titagtagag atgatttggg cagaggecet gggccacetg gaacacatge tictcaagec 961 agtgaacagg attagcetca acgatgtgag caaggcagag gggattetee ttetagtaaa 1021 ggcagcactg aaaaatggag aaacagcaga gcaattgcaa aagatgatga cagagtttta 1081 cagactgata ceteacaaag geacaatgee caaagaagtg aacetgggae tattggetaa 1141 gaaagcagac ctctgccagc taataagaga catggttaat gtctgtgaaa ctaatttgtc 1201 caaacccaac ccaccatece tggccaaata ccgagetttg aggtgcaaaa ttgagcatgt 1261 tgaacagaat actgaagaat ttctcagggt tagaaaagag gttttgcaga atcatcacag 1321 taagagccca gtggatgtet tgcagatatt tagagttggc agagtgaatg aaaccacaga 1381 gtttttgage aaacttggta atgtgaggee ettgttgeat ggtteteetg tacaaaacat 1441 cgtgggaate ttgtgtcgag ggttgetttt acccaaagta gtggaagate gtggtgtgca 1501 aagaacagac gtcggaaacc ttggaagtgg gatttatttc agtgattcgc tcagtacaag 1561 tatcaagtac tcacaccegg gagagacaga tggcaccaga ctcctgctca tttgtgacgt 1621 agccctegga aagtgtatgg acttacatga gaaggacttt ceettaactg aagcaccacc 1681 aggetacgae agtgtgeatg gagttteaea aacagcetet gteaceaeag actttgagga 1741 tgatgaattt gttgtctata aaaccaatca ggttaaaatg aaatatatta ttaaattttc 1801 catgcctgga gatcagataa aggactttca tectagtgat catactgaat tagaggaata 1861 cagacetgag tttteaaatt ttteaaaggt tgaagattae cagttaecag atgecaaaac 1921 ttccagcagc accaaggeeg geetecagga tgeetetggg aacttggtte etetggagga 1981 tgtccacatc aaagggagaa tcatagacac tgtagcccag gtcattgttt ttcagacata 2041 cacaaataaa agtcacgtgc ccattgaggc aaaatatatc tttcctttgg atgacaaggc 2101 cgctgtgtgt ggcttcgaag ccttcatcaa tgggaagcac atagttggag agattaaaga 2161 gaaggaagaa geecagcaag agtacetaga ageegtgace cagggecatg gegettacet 2221 gatgagteag gatgeteegg aegtttttae tgtaagtgtt ggaaaettae cecetaagge 2281 taaggttett ataaaaatta eetacateae agaacteage ateetgggea etgttggtgt 2341 ctttttcatg cocgccaccg tagcaccctg gcaacaggac aaggctttga atgaaaacct 2401 teaggataca gtagagaaga tttgtataaa agaaatagga acaaagcaaa gettetettt 2461 gactatgtct attgagatgc cgtatgtgat tgaattcatt ttcagtgata cacatgaact 2521 gaaacaaaag egeacagact geaaagetgt cattageace atggaaggea geteettaga 2581 cagcagtgga ttttctctcc acatcggttt gtctgctgcc tatctcccaa gaatgtgggt 2641 tgaaaaacat ccagaaaaag aaagegagge ttgeatgett gtettteaac eegatetega 2701 tgtcgacctc cctgacctag ccagtgagag cgaagtgatt atttgtcttg actgctccag

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2761 ttccatggag ggtgtgacat tettgeaage caageaaate acettgeatg egetgteett 2821 ggtgggtgag aagcagaaag taaatattat ccagttcggc acaggttaca aggagctatt 2881 ttegtateet aageatatea caageaatae caeggeagea gagtteatea tgtetgeeae 2941 acctaccatg gggaacacag acttctggaa aacactccga tatcttagct tattgtaccc 3001 tgetcgaggg teacggaaca teeteetggt gtetgatggg caceteeagg atgagageet 3061 gacattacag etegtgaaga ggageegeee geacaceagg ttattegeet geggtategg 3121 ttctacagea aategteaeg tettaaggat ttigteeeag tgtggtgeeg gagtattiga 3181 atattttaat gcaaaatcca agcatagttg gagaaaacag atagaagacc aaatgaccag 3241 getatgitet cegagitigee aetetgiete egicaaatigg cageaactea atecagatige 3301 georgaggee etgeaggeee eageocaggt geoateettg titegeaatg ategacteet 3361 tgtctatgga ttcattcctc actgcacaca agcaactctg tgtgcactaa ttcaagagaa 3421 agaattitgt acaatggtgt egactactga getteagaag acaactggaa etatgateea 3481 caagetggca geeegagete taateagaga ttatgaagat ggeattette acgaaaatga 3541 aaccagteat gagatgaaaa aacaaacett gaaatetetg attattaaac teagtaaaga 3601 aaactetete ataacacaat ttacaagett tgiggcagtt gagaaaaggg atgagaatga 3661 gtcgcctttt cctgatattc caaaagtttc tgaacttatt gccaaagaag atgtagactt 3721 cetgecetae atgagetgge agggggagee ecaagaagee gteaggaace agtetettit 3781 agcatectet gagtggeeag aattaegttt atecaaaega aaacatagga aaattecatt 3841 ttccaaaaga aaaatggaat tatctcagcc agaagtttct gaagattttg aagaggatgg 3901 cttaggtgta ctaccagett teacateaaa tttggaaegt ggaggtgtgg aaaagetatt 3961 ggatttaagt tggacagagt catgtaaacc aacagcaact gaaccactat ttaagaaagt 4021 cagiccatgg gaaacatcta citetagett tittectatt tiggeteegg eegitggite 4081 ctatettace cegactacee gegeteacag teetgettee tigtettitg ceteatateg 4141 teaggtaget agtiteggti eagetgetee teecagacag titgatgeat eteaatteag 4201 ccaaggeet gtgeetggea ettgtgetga etggateeea eagteggegt ettgteeeae 4261 aggacetece cagaaceeae citetgeace etattgtgge attgttttt cagggagete 4321 attaagetet geaeagtetg eteeactgea acateetgga ggetttaeta eeaggeette 4381 tgetggeaec ttecetgage tggattetee ceagetteat ttetetette etacagaece 4441 tgateceate agaggttttg ggtettatea teeetetget tacteteett ticattttea 4501 acetteegea geetetttga etgecaaeet taggetgeea atggeetetg etttaeetga 4561 ggetetttge agteagteee ggactaceee agtagatete tgtettetag aagaateagt 4621 aggeagtete gaaggaagte gatgteetgt etttgetttt caaagttetg acacagaaag 4681 tgatgageta teagaagtae tteaagaeag etgettttta caaataaagt gtgatacaaa 4741 agatgacagt atcccgtgct ttctggaatt aaaagaagag gatgaaatag tgtgcacaca 4801 acactggcag gatgetgtgc cttggaeaga acteeteagt etacagaeag aggatggett 4861 etggaaactt acaccagaac tgggacttat attaaatett aatacaaatg gtttgcacag 4921 ctttettaaa eaaaaaggea tteaatetet aggtgtaaaa ggaagagaat gteteetgga 4981 cctaattgcc acaatgctgg tactacagtt tattcgcacc aggttggaaa aagagggaat 5041 agtgitcaaa tcactgatga aaatggatga cectictatt tccaggaata ttccctgggc 5101 tittgaggca ataaagcaag caagtgaatg ggtaagaaga actgaaggac agtacccatc 5161 tatetgeeca eggettgaae tggggaaega etgggaetet geeaceaage agttgetggg 5221 actoragece ataageaetg tgteceetet teatagagte etecattaca gteaaggeta 5281 agtcaaatga aactgaattt taaacttttt geatgettet aigtagaaaa taatcaaatg 5341 ataatagata attataatga aacticatta aggitteatt eagtgfagea attactgtet 5401 ttaaaaatta agtggaagaa gaattacttt aatcaactaa caagcaataa taaaatgaaa 5461 cttaaaataa aaaaaaaaaa aaaaaaaaaa

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USE OF RNAI INHIBITING PARP ACTIVITY FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF CANCER

This invention relates to the use of an agent that inhibits the activity of an enzyme which mediates the repair of DNA strand breaks in the treatment of certain forms of cancer in particular breast cancer.

Homologous recombination (HR) has been shown to play an important role in repair of damage occurring at DNA replication forks in mammalian cells (2). Thus, cells deficient in HR show retarded growth and exhibit higher level of genetic instability. It is believed that genetic instability due to loss of HR repair in human cancers significantly contributes to the development of cancer in these cells (1).

Post transcriptional modification of nuclear proteins by poly(ADP-ribosyl)ation (PARP) in response to DNA strand breaks plays an important role in DNA repair, regulation of 20 apoptosis, and maintenance of genomic stability.

Poly(ADP-ribose)Polymerase (PARP-1) is an abundant nuclear protein in mammalian cells that catalyses the formation of poly(ADP-ribose) (PAR) polymers using NAD⁺ as substrate. Upon DNA damage, PARP-1 binds rapidly to a 25 DNA strand break (single strand or double strand) and catalyses the addition of negatively charged PAR chains to itself (automodification) and other proteins (see [3, 4] for reviews). The binding of PARP-1 to DNA strand breaks is believed to protect DNA lesions from further processing until PARP-1 is 30 dissociated from the break by the accumulated negative charge resulting from PAR polymers (5,6).

Although PARP-1 has been implicated in several nuclear processes, such as modulation of chromatin structure, DNA replication, DNA repair and transcription, PARP-1 knockout 35 mice develop normally (7). Cells isolated from these mice exhibit a hyper recombination phenotype and genetic instability in the form of increased levels of SCE, micronuclei and tetraploidy (8-10). Genetic instability may also occur in these PARP-1 knockout mice through telomere shortening, 40 increased frequency of chromosome fusion and aneuploidy (11), although all of these results could not be repeated in another set of PARP-1 knock-out mice (12). In the former mice knockout, PARP-1 null mutation rescue impaired V(D)J recombination in SCID mice (13).

These results support the view suggested by Lindahl and coworkers that PARP-1 has a protective role against recombination (5). They proposed that binding of PARP-1 to DNA strand breaks prevents the recombination machinery from recognizing and processing DNA lesions or, alternatively, 50 that the negative charges accumulated following poly ADP-ribosylation repel adjacent recombinogenic DNA sequences. Only the latter model is consistent with inhibition of PARP-1 itself and expression of a dominant negative mutant PARP-1, inducing SCE, gene amplification and homologous recombination (HR [14-18]).

Studies based on treating cells with PARP inhibitors or cells derived from PARP-1 or PARP-2 knockout mice indicate that the suppression of PARP-1 activity increases cell susceptibility to DNA damaging agents and inhibits strand 60 break rejoining (3, 4, 8-11, 19, 20, 47).

Inhibitors of PARP-1 activity have been used in combination with traditional anti-cancer agents such as radio therapy and chemotherapy (21). The inhibitors were used in combination with methylating agents, topoisomerase poisons and 65 ionising radiations and were found to enhance the effectiveness of these forms of treatment. Such treatments, however, 2

are known to cause damage and death to non cancerous or "healthy" cells and are associated with unpleasant side effects

There is therefore a need for a treatment for cancer that is both effective and selective in the killing of cancer cells and which does not need to be administered in combination with radio or chemotherapy treatments.

The present inventors have surprisingly found that cells deficient in homologous recombination (HR) are hypersensitive to PARP inhibitors as compared to wild type cells. This is surprising since PARP-1 knockout mice live normally thereby indicating that PARP-1 is not essential for life. Thus, it could not be expected that cells would be sensitive to PARP inhibition.

According to a first aspect of the invention there is provided the use of an agent that inhibits the activity of an enzyme that mediates the repair of DNA strand breaks in the manufacture of a medicament for the treatment of diseases that are caused by a genetic defect in a gene that mediates homologous recombination.

In a further aspect the invention provides a method of treatment of a disease or condition in a mammal, including human, which is caused by a genetic defect in a gene which mediates homologous recombination, which method comprises administering to the mammal a therapeutically effective amount of an agent which inhibits the activity of an enzyme which mediates repair of DNA strand breaks or other lesions present at replication forks.

In a preferred aspect said enzyme is PARP. In a further preferred aspect said agent is a PARP inhibitor or an RNAi molecule specific to PARP gene.

In a further preferred aspect, the use is in the treatment of cancer.

Preferably the medicament is a pharmaceutical composition consisting of the PARP inhibitor in combination with a pharmaceutically acceptable carrier or diluent.

The specific sensitivity of HR defective tumours to PARP-1 inhibition means that normally dividing cells in the patient will be unaffected by the treatment. Treatment of HR defective cancer cells using a PARP inhibitor also has the advantage that it does not need to be administered as a combination therapy along with conventional radio or chemotherapy treatments thereby avoiding the side effects associated with these conventional forms of treatment.

A genetic defect in a gene which mediates homologous recombination may be due to a mutation in, the absence of, or defective expression of, a gene encoding a protein involved in HR.

In a further aspect, the invention further provides the use of a PARP inhibitor in the manufacture of a medicament for inducing apoptosis in HR defective cells.

In another aspect the invention provides a method of inducing apoptosis in HR defective cells in a mammal which method comprises administering to the mammal a therapeutically effective amount of a PARP inhibitor.

By causing apoptosis in HR defective cells it should be possible to reduce or halt the growth of a tumour in the mammal.

Preferably, the HR defective cells are cancer cells.

Cancer cells defective in HR may partially or totally deficient in HR. Preferably the cancer cells are totally deficient in HR.

The term "cancer" or "tumour" includes lung, colon, pancreatic, gastric, ovarian, cervical, breast or prostate cancer. The cancer may also include skin, renal, liver, bladder or cerebral cancer. In a preferred aspect, the cancer is in a mammal, preferably human.

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The cancer to be treated may be an inherited form of cancer wherein the patient to be treated has a familial predisposition to the cancer. Preferably, the cancer to be treated is genelinked hereditary cancer. In a preferred embodiment of the invention the cancer is gene-linked hereditary breast cancer.

In a preferred aspect, the PARP inhibitor is useful in the treatment of cancer cells defective in the expression of a gene involved in HR. Genes with suggested function in HR include XRCC1, ADPRT (PARP-1), ADPRTL2 (PARP-2), CTPS, RPA, RPA1, RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51, RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, BRCA1, BRCA2, RAD52, RAD54, RAD50, MRE11, NBS1, WRN, BLM, Ku70, Ku80, ATM, ATR, chk1, chk2, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, RAD1, RAD9, FEN-1, Mus81. Eme1, DDS1, BARD (see (2, 3, 5, 22-28) for reviews).

A gene involved in HR may be a tumour suppressor gene. The invention thus provides for the treatment of cancer cells defective in the expression of a tumour suppressor gene. 20 Preferably, the tumour suppressor gene is BRCA1 or BRCA2.

Breast cancer is the most common cancer disease among women in the Western world today. Certain families have strong predisposition for breast cancer, which is often owing 25 to an inherited mutation in one allele of either BRCA1 or BRCA2. However, these patients still maintain one functional allele. Thus, these patients develop normally and have no phenotypic consequence from this mutation. However, in one cell, the functional allele might be lost, making this cell 30 cancerous and at the same time deficient in homologous recombination (HR). This step is critical for the onset of a tumour (1).

The present inventors have surprisingly found that BRCA2 deficient cells are 100 times more sensitive to the cytotoxicity 35 of the PARP inhibitor, NU1025, than wild type cells.

Thus in a preferred aspect, the invention provides the use of a PARP inhibitor in the manufacture of a medicament for the treatment of cancer cells defective in HR, e.g due to the loss of BRCA1 and/or BRCA2 expression.

The cancer cells to be treated may be partially or totally deficient in BRCA1 or BRCA2 expression. BRCA1 and BRCA2 mutations can be identified using multiplex PCR techniques, array techniques (29, 30) or using other screens known to the skilled person.

PARP inhibitors useful in the present invention may be selected from inhibitors of PARP-1, PARP-2, PARP-3, PARP-4, tankyrase 1 or tankyrase 2 (see 31 for a review). In a preferred embodiment, the PARP inhibitor useful in the present invention is an inhibitor of PARP-1 activity.

PARP inhibitors useful in the present invention include benzimidazole-carboxamides, quinazolin-4-[3H]-ones and isoquinoline derivatives (e.g. 2-(4-hydroxyphenyl)benzimidazole-4-carboxamide (NU1085), 8-hydroxy-2-methylquinazolin-4-[3H]one (NU1025); 6(5H)phenanthridinone; 55 aminobenzamide; benzimidazole-4-carboxamides (BZ1-6) and tricyclic lactam indoles (TI1-5)[32]. Further inhibitors of PARP may be identified either by design [33] or the novel FlashPlate assay [34].

The PARP inhibitor formulated as a pharmaceutical composition may be administered in any effective, convenient manner effective for targeting cancer cells including, for instance, administration by oral, intravenous, intramuscular, intradermal, intranasal, topical routes among others. Carriers or diluents useful in the pharmaceutical composition may 65 include, but are not limited to saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof.

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In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion. The inhibitor may be administered directly to a tumour or may be targeted to the tumour via systemic administration.

A therapeutically effective amount of the inhibitor is typically one which is sufficient to achieve the desired effect and may vary according to the nature and severity of the disease condition, and the potency of the inhibitor. It will be appreciated that different concentrations may be employed for prophylaxis than for treatment of an active disease.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be up to 100 mg/kg, for example from 0.01 mg/kg to 50 mg/kg body weight, typically up to 0.1, 0.5, 1.0, 2.0 5.0, 10, 15, 20 or 30 mg/kg body weight. Ultimately, however, the amount of inhibitor administered and the frequency of administration will be at the discretion of a physician.

A therapeutic advantage of using PARP inhibitors to treat cancer cells is that only very low doses are needed to have a therapeutic effect in treating cancer thereby reducing systemic build up of the inhibitors and any associated toxic effects

A preferred aspect of the invention provides an agent which is an inhibitory RNA (RNAi) molecule.

A technique to specifically ablate gene function is through the introduction of double stranded RNA, also referred to as inhibitory RNA (RNAi), into a cell which results in the destruction of mRNA complementary to the sequence included in the RNAi molecule. The RNAi molecule comprises two complementary strands of RNA (a sense strand and an antisense strand) annealed to each other to form a double stranded RNA molecule. The RNAi molecule is typically derived from exonic or coding sequence of the gene which is to be ablated.

Preferably said RNAi molecule is derived from the nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

- a) a nucleic acid sequence as represented by the sequence in FIG. 9, 10, 11, 12, 13 or 14 or fragment thereof;
- b) a nucleic acid sequence which hybridises to the nucleic acid sequences of FIG. 9, 10, 11, 12, 13 or 14 and encodes a gene for PARP;
- c) a nucleic acid sequence which comprise sequences which are degenerate as a result of the genetic code to the nucleic acid sequences defined in (a) and (b).

Recent studies suggest that RNAi molecules ranging from 100-1000 bp derived from coding sequence are effective inhibitors of gene expression. Surprisingly, only a few molecules of RNAi are required to block gene expression which implies the mechanism is catalytic. The site of action appears to be nuclear as little if any RNAi is detectable in the cytoplasm of cells indicating that RNAi exerts its effect during 55 mRNA synthesis or processing.

More preferably said RNAi molecule according has a length of between 10 nucleotide bases (nb)-1000 nb. Even more preferably said RNAi molecule has a length of 10 nb; 20 nb; 30 nb; 40 nb; 50 nb; 60 nb; 70 nb; 80 nb; 90 nb; or 100 bp. Even more preferably still said RNAi molecule is 21 nb in length.

Even more preferably still the RNAi molecule comprises the nucleic acid sequence aaa agc cau ggu gga gua uga (PARP-1)

Even more preferably still the RNAi molecule consists of the nucleic acid sequence aag acc aau cuc ucc agu uca ac (PARP-2)

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Even more preferably still the RNAi molecule consists of the nucleic acid sequence aag acc aac auc gag aac aac (PARP-

The RNAi molecule may comprise modified nucleotide bases

Preferred features of each aspect of the invention are as for each of the other aspects mutatis mutandis.

The present invention will now be described by way of example only with reference to the accompanying figures, wherein:

FIG. 1 is a graph demonstrating that HR deficient cells are hypersensitive to the toxic effect caused by inhibition of PARP-1. Colony outgrowth of the Chinese hamster cell lines AA8 (wild-type), irs1SF (deficient in HR[4]), CXR3 (irs1SF complemented with XRCC3 [2]), V79 (wild-type), irs1 (defi-15 cient in HR[5]) or irs1X2.2 (irs1 complimented with XRCC2 [1]) upon exposure to 3-AB (A), ISQ (B) or NU1025 (C). The means (symbols) and standard deviation (bars) of at least three experiments are shown. Colony outgrowth assay was used:

FIG. 2 is a graph showing cell survival in the presence of PARP inhibitor NU1025 in wt V79 cells, BRCA2 deficient VC-8 cells and VC-8 cells complimented with functional BRCA2 gene (VC-8#13, VC-8+B2). Colony outgrowth assay was used;

FIG. 3 is a histogram showing the percentage of the cells in apoptosis following a 72 hour incubation with NU1025;

FIG. 4. (a) Western blot analysis of protein lysates isolated from MCF-7 (p53^{wt}) or MDA-MB-231 (p53^{mut}) breast cancer cells following 48 hours transfection with siRNA. (b) 30 Colony outgrowth of siRNA-treated MCF-7 cells or (c) MDA-MB-231 cells following exposure to the PARP inhibitor NU1025. The means (symbols) and standard deviation (bars) of at least three experiments are shown.

FIG. 5. BRCA2 deficient cells fail to repair a recombina- 35 tion lesion formed at replication forks by inhibitors of PARP. (a) Visualization of double strand breaks (DSBs) in BRCA2 proficient or deficient cells following a 24-hour treatment with NU1025 (0.1 mM) by pulse-field gel electrophoresis. Hydroxyurea 2 mM was used as a positive control. (b) Visu- 40 alisation of yH2Ax foci in untreated V-C8+B2 and V-C8 cells. Number of cells containing yH2Ax foci (c) or RAD51 foci (d) visualised in V-C8+B2 and V-C8 cells following a 24-hour treatment with NU1025 (10 µM). The means (symbols) and standard errors (bars) of three to nine experiments are shown. 45 (e) A suggested model for cell death induced in BRCA2 deficient cells.

FIG. 6. PARP-1 and not PARP-2 is important in preventing formation of a recombinogenic lesion, causing death in absence of BRCA2. (a) RT-PCR on RNA isolated from 50 SW480SN.3 cells treated with BRCA2, PARP-1 and PARP-2 siRNA in combinations as shown for 48 hours. (b) Clonogenic survival following 48-hours depletion of BRCA2, PARP-1 and PARP-2. The means (symbols) and standard deviation (bars) of at least three experiments are shown. Two 55 and three stars designate statistical significance in t-test p<0.01 and p<0.001, respectively. (c) Western blot for PARP-1 in SW480SN.3 cells treated with different siRNA.

FIG. 7. (a) Visualisation of PAR polymers in untreated and (b) thymidine treated V79 cells (5 mM for 24 hours). (c) 60 Percentage cells containing >10 sites of PARP activity following treatment with hydroxyurea (0.2 mM) and thymidine (5 mM). At least 300 nuclei were counted for each treatment and experiment. (d) Survival of V-C8+B2 cells following co-treatment with hydroxyurea or (e) thymidine and NU1025 (10 µM). (f) The activity of PARP was measured by the level of free NAD(P)H¹¹, following treatment with MMS, hydrox-

yurea (0.5 mM) or thymidine (10 mM). The means (symbol) and standard deviation (error bars) from at least three experiments are depicted.

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FIG. 8. (a) Visualisation of PAR polymers in untreated 5 V-C8 and (b) V-C8+B2 cells. (c) Quantification of percentage cells containing >10 sites of PARP activity in untreated V-C8 and V-C8+B2 cells. (d) Level of NAD(P)H measured in untreated V-C8 and V-C8+B2 cells. Three stars designate p<0.001 in t-test. (e) Visualization of RAD51 and sites of PARP activity in V79 cells following a 24-hour thymidine treatment (5 mM). (f) A model for the role of PARP and HR at stalled replication forks.

FIG. 9 is the human cDNA sequence of PARP-1;

FIG. 10 is the human cDNA sequence of PARP-2;

FIG. 11 is the human cDNA sequence of PARP-3;

FIG. 12 is the human gDNA sequence of Tankyrase 1;

FIG. 13 is the human mRNA sequence of Tankyrase 2;

FIG. 14 is the human mRNA sequence of VPARP.

MATERIALS AND METHODS

Cytotoxicity of PARP Inhibitors to HR-Defective Cells: XRCC2, XRCC3 or BRCA2

Cell Culture

The irs1, irs1X2.1 and V79-4 cell lines were a donation from John Thacker [40] and the AA8, irs1SF and CXR3 cell lines were provided by Larry Thompson [41].

The VC-8, VC-8+B2, VC-8#13 were a gift from Malgorzata Zdzienicka [42]. All cell lines in this study were grown in Dulbecco's modified Eagle's Medium (DMEM) with 10% Foetal bovine serum and penicillin (100 U/ml) and streptomycin sulphate (100 µg/mL) at 37° C. under an atmosphere containing 5% CO₂.

Toxicity Assay—Colony Outgrowth Assay

500 cells suspended in medium were plated onto a Petri dish 4 hours prior to the addition of 3-AB, ISQ or NU1025. ISQ and NU1025 were dissolved in DMSO to a final concentration of 0.2% in treatment medium. 7-12 days later, when colonies could be observed, these colonies were fixed and stained with methylene blue in methanol (4 g/l). Colonies consisting of more than 50 cells were subsequently counted.

Apoptosis Experiments

 0.25×10^6 cells were plated onto Petri dishes and grown for 4 hours before treatment with NU1025. After 72 hours, cells were trypsinized and resuspended with medium containing any floating cells from that sample. The cells were pelleted by centrifugation and resuspended for apoptosis analysis with FITC-conjugated annexin-V and propidium iodine (PI) (ApoTarget, Biosource International) according to manufacturer's protocol. Samples were analysed by flow cytometry (Becton-Dickenson FACSort, 488 nm laser), and percentage of apoptotic cells was determined by the fraction of live cells (PI-negative) bound with FITC-conjugated annexin-V.

Immunofluorescence

Cells were plated onto coverslips 4 h prior to 24-h treatments as indicated. Following treatments the medium was removed and coverslips rinsed once in PBS at 37° C. and fixed as described elsewhere [2]. The primary antibodies and dilutions used in this study were; rabbit polyclonal anti PAR (Trevigen; 1:500), goat polyclonal anti Rad51 (C-20, Santa Cruz; 1:200) and rabbit polyclonal anti Rad51 (H-92, Santa Cruz; 1:1000). The secondary antibodies were Cy-3-conjugated goat anti-rabbit IgG antibody (Zymed; 1:500), Alexa 555 goat anti-rabbit F(ab')₂IgG antibody (Molecular Probes; 1:500), Alexa 546 donkey anti-goat IgG antibody (Molecular Probes; 1:500) and Alexa 488 donkey anti-rabbit IgG antibody (Molecular Probes; 1:500). Antibodies were diluted in

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PBS containing 3% bovine serum albumin. DNA was stained with 1 $\mu g/ml$ To Pro (Molecular Probes). Images were obtained with a Zeiss LSM 510 inverted confocal microscope using planapochromat 63X/NA 1.4 oil immersion objective and excitation wavelengths 488, 546 and 630 nm. Through focus maximum projection images were acquired from optical sections 0.50 μm apart and with a section thickness of 1.0 μm . Images were processed using Adobe PhotoShop (Abacus Inc). At least 300 nuclei were counted on each slide and those containing more than 10 RAD51 foci or sites of PARP activity were classified as positive.

PARP Activity Assays

A water-soluble tetrazolium salt (5 mM WST-8) was used to monitor the amount of NAD(P)H through its reduction to a yellow coloured formazan dye[43]. 5000 cells were plated in at least triplicate into wells of a 96 well plate and cultured in 100 μl normal growth media for 4 h at 37° C. CK8 buffer (Dojindo Molecular Technology, Gaithersburg, USA), containing WST-8, was then added either with or without treatment with DNA damaging agents at concentrations indicated. Reduction of WST-8 in the presence of NAD(P)H was deter- 20 mined by measuring visible absorbance (OD_{450}) every 30 min. A medium blank was also prepared containing just media and CK8 buffer. Changes in NAD(P)H levels were calculated by comparing the absorbance of wells containing cells treated with DNA damaging agents and those treated with DMSO alone. Alternately relative levels of NAD(P)H in different cells lines were calculated after 4 h incubation in

The ability of NU1025 to inhibit PARP-1 activity was also assayed in permeabilised cells using a modification of the method of Halldorsson et al [44], and described in detail elsewhere [45]. Briefly: 300 μ l of NU1025-treated (15 min) permeabilised cells were incubated at 26° C. with oligonucleotide (final conc. 2.5 μ g/ml), 75 μ M NAD+[32 P] NAD (Amersham Pharmacia, Amersham, UK) in a total volume of 400 μ l. The reaction was terminated after 5 min by adding ice cold 10% TCA 10% Na Ppi for 60 min prior to filtering through a Whatman GF/C filter (LabSales, Maidstone, UK), rinsed 6x with 1% TCA 1% NaPPi, left to dry and incorporated radioactivity was measured to determine PARP-1 activity. Data are expressed as pmol NAD incorporated/106 cells by reference 40 to [32 P] NAD standards.

Pulse-Field Gel Electrophoresis

 1.5×10^6 cells were plated onto 100 mm dishes and allowed 4 h for attachment. Exposure to drug was for 18 h after which cells were trypsinsied and 10^6 cells melted into each 1% agarose insert. These inserts were incubated as described elsewhere (8) and separated by pulse-field gel electrophoresis for 24 h (BioRad; 120° angle, 60 to 240 s switch time, 4 V/cm). The gel was subsequently stained with ethidium bromide for analysis.

siRNA Treatment

Predesigned BRCA2 SMARTpool and scrambled siRNAs were purchased (Dharmacon, Lafayette, Colo.). 10000 cells seeded onto 6 well plates and left over night before transfected with 100 nM siRNA using Oligofectamine Reagent (Invitrogen) according to manufacturers instructions. Cells were then cultured in normal growth media for 48 h prior to trypsinisation and replating for toxicity assays. Suppression of BRCA2 was confirmed by Western blotting (as described previously [46]) of protein extracts treated with siRNA with an antibody against BRCA2 (Oncogene, Nottingham, UK). 60

EXAMPLES

Homologous Recombination Deficient Cells are Hypersensitive to PARP-1 Inhibition

To investigate the involvement of HR in cellular responses to inhibition of PARP-1, the effects of PARP-1 inhibitors on 8

the survival of HR repair deficient cell lines were studied. It was found that cells deficient in HR (i.e., irs1SF which is defective in XRCC3 or irs1 which is defective in XRCC2 [see Table 1] were very sensitive to the toxic effect of 3-aminobenzamide (3-AB) and to two more potent inhibitors of PARP-1: 1,5-dihydroxyisoquinoline (ISQ; [37]) or 8-hydroxy-2-methylquinazolinone (NU1025 [38, 39]) (FIG. 1). The sensitivity in irs1SF cells to 3-AB, ISQ or NU1025 was corrected by the introduction of a cosmid containing a functional XRCC3 gene (CXR3). Similarly, the sensitivity in irs1 cells to 3-AB, ISQ or NU1025 was corrected by the introduction of a cosmid containing a functional XRCC2 gene (irs1X2.2).

BRCA2 Deficient Cells are Hypersensitive to PARP-1 Inhibition

The survival of BRCA2 deficient cells (VC8) and wild type cells (V79Z) in the presence of inhibitors of PARP-1 was investigated. It was found that VC8 cells are very sensitive to the toxic effect of NU1025 (FIG. 2). The sensitivity in VC8 cells was corrected by the introduction of a functional BRCA2 gene either on chromosome 13 (VC8#13) or on an overexpression vector (VC8+B2). This result demonstrates that the sensitivity to PARP-1 inhibitors is a direct consequence of loss of the BRCA2 function.

To investigate if inhibition of PARP-1 triggers apoptosis in BRCA2 deficient cells, the level of apoptosis 72 hours following exposure to NU1025 was investigated. It was found that NU1025 triggered apoptosis only in VC8 cells, showing that loss of PARP-1 activity in BRCA2 deficient cells triggers this means of death (FIG. 3).

BRCA2 Deficient Breast Cancer Cells are Hypersensitive to PARP-1 Inhibition

It was examined whether the MCF7 (wild-type p53) and MDA-MB-231 (mutated p53) breast cancer cell lines displayed a similar sensitivity to NU1025 upon depletion of BRCA2. It was found that PARP inhibitors profoundly reduced the survival of MCF7 and MDA-MB-231 cells only when BRCA2 was depleted with a mixture of BRCA2 siRNA (FIG. 4). This shows that BRCA2 depleted breast cancer cells are sensitive to PARP inhibitors regardless of p53 status.

BRCA2 Deficient Cells Die from PARP-1 Inhibition in Absence of DNA Double-Strand Breaks (DSBs) but in Presence of γH2Ax

HR is known to be involved in the repair of DSBs and other lesions that occur during DNA replication [2]. To determine whether the sensitivity of BRCA2 deficient cells is the result of an inability to repair DSBs following NU1025 treatment, the accumulation of DSBs in V79 and V-C8 cells was measured following treatments with highly toxic levels of NU1025. It was found that no DSBs were detectable by pulsed field gel electrophoretic analysis of DNA obtained from the treated cells (FIG. 5A), suggesting that low levels of DSBs or other recombingenic substrates accumulated following PARP inhibition in HR deficient cells, which trigger γH2Ax FIG. 5B). The reason why BRCA2 deficient cells die following induction of these recombinogenic lesions is likely to be due to an inability to repair such lesions. To test this, the ability of BRCA2 deficient V-C8 cells and BRCA2 complimented cells to form RAD51 foci in response to NU1025 was determined. It was found that RAD51 foci were indeed induced in V-C8+B2 cells following treatment with NU1025 (statistically significant in t-test p<0.05; FIG. 5D). This indicates that the recombinogenic lesions trigger HR repair in these cells allowing them to survive. In contrast, the BRCA2 deficient V-C8 cells were unable to form RAD51 foci in response to NU1025 treatment (FIG. 5D) indicating no HR, which would leave the recombinogenic lesions unrepaired and thus cause cell death.

PARP-1 and Not PARP-2 is Important in Preventing Formation of a Recombingenic Lesion

There are two major PARPs present in the nucleus in mammalian cells, PARP-1 and PARP-2 and all reported PARP inhibitors inhibit both. In order to distinguish which PARP 5 was responsible for the effect, we tested if the absence of PARP-1 and/or PARP-2 results in accumulation of toxic lesions, by depleting these and BRCA2 with siRNA in human cells (FIG. 6a). We found that the clonogenic survival was significantly reduced when both PARP-1 and BRCA2 pro- 10 teins were co-depleted from human cells (FIG. 6b). Depletion of PARP-2 with BRCA2 had no effect on the clonogenic survival and depletion of PARP-2 in PARP-1 and BRCA2 depleted cells did not result in additional toxicity. These results suggest that PARP-1 and not PARP-2 is responsible 15 for reducing toxic recombinogenic lesions in human cells. The cloning efficiency was only reduced to 60% of control in PARP-1 and BRCA2 co-depleted cells, while no HR deficient cells survived treatments with PARP inhibitors. This is likely to do with incomplete depletion of the abundant PARP-1 20 protein by siRNA (FIG. 6c), which might be sufficient to maintain PARP-1 function in some of the cells.

PARP-1 is Activated by Replication Inhibitors

HR is also involved in repair of lesions occurring at stalled replication forks, which may not involve detectable DSBs [2]. 25 To test if PARP has a role at replication forks, PARP activation in cells treated cells with agents (thymidine or hydroxyurea) that retard or arrest the progression of DNA replication forks was examined. Thymidine depletes cells of dCTP and slows replication forks without causing DSBs. Hydroxyurea 30 depletes several dNTP and block the replication fork, which is associated with the formation of DSBs at replication forks [2]. Both of these agents potently induce HR [2]. V79 hamster cells treated for 24 hours with thymidine or hydroxyurea were stained for PAR polymers. This revealed a substantial 35 increase in the number of cells containing sites of PARP activity (FIG. 7C). This result suggests a function for PARP at stalled replication forks. It was also shown that inhibition of PARP with NU1025 enhances the sensitivity to thymidine or hydroxyurea in V-C8+B2 cells (FIG. 7D,E). This result sug- 40 gests that PARP activity is important in repair of stalled replication forks or alternatively that it prevents the induction of death in cells with stalled replication forks.

PARP is rapidly activated at DNA single-strand breaks (SSB) and attracts DNA repair enzymes [3-6]. Methylmethane sulphonate (MMS) causes alkylation of DNA, which is repaired by base excision repair. PARP is rapidly activated by the SSB-intermediate formed during this repair, which depletes the NAD(P)H levels (FIG. 7F). We found that the activation of PARP and reduction of NAD(P)H levels is 50 much slower following thymidine or hydroxyurea treatments. This slow PARP activation can be explained by the indirect action of thymidine and hydroxyurea and the time required to accumulate stalled replication forks as cells enter the S phase of the cell cycle.

PARP-1 and HR Have Separate Roles at Stalled Replication Forks

The number sites of PARP activity in untreated BRCA2 deficient V-C8 cells was determined. It was found that more V-C8 cells contain sites of PARP activity compared to V-C8+60 B2 cells (FIG. 8A,B,C). Also, the V-C8 cells have lower free NAD(P)H levels than the corrected cells (FIG. 8D), as a likely result of the increased PARP activity. Importantly these sites of PARP activity do not overlap with RAD51 foci (FIG. 8E).

The results herein suggest that PARP and HR have separate 65 roles in the protection or rescue of stalled replication forks (FIG. 8F). A loss of PARP activity can be compensated by

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increased HR while a loss of HR can be compensated by increased PARP activity. However, loss of both these pathways leads to accumulation of stalled replication forks and to death, as in the case of PARP inhibited BRCA2 deficient cells

As shown in the model outlined in FIG. 8F PARP and HR have complementary roles at stalled replication forks. (i) Replication forks may stall when encountering a roadblock on the DNA template. In addition, they may also stall temporarily, due to lack of dNTPs or other replication co-factors. (ii) PARP binds stalled replication forks or other replicationassociated damage, triggering PAR polymerization. Resulting negatively charged PAR polymers may protect stalled replication forks, by repelling proteins that normally would process replication forks (e.g., resolvases), until the replication fork can be restored spontaneously when dNTPs or other co-factors become available. Alternatively, PAR polymers or PARP may attract proteins to resolve the replication block by other means. (iii) In absence of PARP activity, HR may be used as an alternative pathway to repair stalled replication forks. This compensatory model explains the increased level of HR and RAD51 foci found in PARP deficient cells3-5 and higher PARP activity found in HR deficient cells (i.e. V-C8). Spontaneous replication blocks/lesions are only lethal in the absence of both PARP and HR.

TABLE 1

Genotype and origin of cell lines used in this study.					
	Cell line	Genotype	Defect	Origin	Reference
	AA8	Wt	Wt	СНО	[41]
	irs1SF	XRCC3-	XRCC3-, deficient in HR	AA8	[41]
	CXR3	XRCC3 ⁻ +	Wt	irs1SF	[41]
•		hXRCC3			
	V79-4	Wt	Wt	V79	[40]
	irs1	XRCC2-	XRCC2 ⁻ , deficient in HR	V79-4	[40]
	irs1X2.2	XRCC2 ⁻ +	Wt	irs1	[40]
		hXRCC2			
	V79-Z	Wt	Wt	V79	[42]
)	VC8	BRCA2	BRCA2 ⁻ , deficient in HR	V79-Z	[42]
	VC8#13	BRCA2" +	Wt	VC8	[42]
		hBRCA2			
	VC8 + B2	BRCA2 ⁻ +	Wt	VC8	[42]
		hBRCA2			

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37 38 -continued acactggcag gatgctgtgc cttggacaga actcctcagt ctacagacag aggatggctt ctggaaactt acaccagaac tgggacttat attaaatctt aatacaaatg gtttgcacag 4920 ctttcttaaa caaaaaggca ttcaatctct aggtgtaaaa ggaagagaat gtctcctgga 4980 cctaattgcc acaatgctgg tactacagtt tattcgcacc aggttggaaa aagagggaat 5040 agtgttcaaa tcactgatga aaatggatga cccttctatt tccaggaata ttccctgggc 5100 ttttgaggca ataaagcaag caagtgaatg ggtaagaaga actgaaggac agtacccatc 5160 tatctgccca cggcttgaac tggggaacga ctgggactct gccaccaagc agttgctggg actocagoco ataagoactg tgtcccctct tcatagagtc ctccattaca gtcaaggcta 5280 agtcaaatga aactgaattt taaacttttt gcatgcttct atgtagaaaa taatcaaatg ataatagata attataatga aacttcatta aggtttcatt cagtgtagca attactgtct ttaaaaatta agtggaagaa gaattacttt aatcaactaa caagcaataa taaaatgaaa 5460

The invention claimed is:

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1. A method of treatment of cancer cells defective in homologous recombination (HR), the method comprising: identifying a human patient with a familial predisposition to gene-linked hereditary cancer, wherein said cancer comprises cancer cells defective in homologous recombination;

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identifying a compound which inhibits PARP-1, and administering to said human patient a therapeutically effective amount of said compound.

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